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DNA Adducts in Human Tissues: Biomarkers of Exposure to Carcinogens in Tobacco Smoke

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Tobacco smoking causes millions of cancer deaths annually. Tobacco smoke is a complex mixture of thousands of chemicals including many known animal carcinogens. Because many carcinogens form DNA adducts in target animal or human tissues, the detection of the formation of adducts using such methods as postlabeling, immunoassay, fluorescence spectroscopy, and mass spectrometry is a means of monitoring human exposure to tobacco carcinogens. Smokers are at increased risk of cancer in many organs, and studies have revealed either specific adducts related to smoking or increased levels of adducts in the lung, bronchus, larynx, bladder, cervix, and oral mucosa of smokers. In a limited number of studies, the adducts and the carcinogens responsible for them have been identified. Some studies have demonstrated higher levels of adducts in the white blood cells of smokers, while other studies indicate other sources of genotoxic agents, including diet, can contribute to the DNA damage observed in these cells. — *Environ Health Perspect* 104(Suppl 3):453–458 (1996)

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Tobacco Use and Tobacco-related Diseases

Tobacco smoking and related uses are a major cause of human disease. Cigarette smoking is causally related to cancer of the respiratory tract, upper digestive tract, pancreas, renal pelvis, and bladder (1,2). Cigar and pipe smoking are causally related to cancer of the respiratory tract, oral cavity, and esophagus. The use of chewing tobacco and oral snuff is associated with cancer of the oral cavity, kidney, and bladder (3). Passive smoking has also been widely implicated as a risk factor for lung cancer in nonsmokers. In addition to cancer, smokers are at increased risk of stroke, heart attack, peripheral vascular disease,

aortic aneurysm, emphysema and bronchitis, and, in old age, cataract and delayed bone healing. Smoking also causes reduced fertility, excessive bleeding during pregnancy, and lower infant birth weight (1).

Prior to the twentieth century, tobacco use was largely confined to pipe and cigar smoking among a small section of the population. The emergence of tobacco use as a major cause of death and disease is the result of the growth in popularity of cigarettes, aided by the development of machines for their mass production and the invention of safety matches. Although there has been a leveling, or even a decline, in tobacco consumption in some Western countries in the last few years, the global trend is still one of increasing use. Cigarette production worldwide is estimated to have increased by 2.2% annually in the last 20 years, compared with an annual growth in world population of 1.7%. It is predicted that this trend will accelerate to 2.9% a year throughout the 1990s (1).

Carcinogens in Tobacco Smoke

Tobacco smoke is a very complex mixture of materials, containing around 400 to 500 gaseous components and 1×10^{10} particles/ml (4). More than 3,500 chemicals

make up the particulate phase and, overall, at least 43 chemicals that have been demonstrated to be animal carcinogens have been detected in tobacco smoke. In addition, tobacco smoke contains many free radicals—those in the gaseous phase being short-lived and those in the particulate phase relatively long-lived. In general, sidestream smoke (also known as environmental tobacco smoke, which is inhaled by nonsmokers) contains higher levels of identified carcinogens than mainstream smoke (that which is directly inhaled by smokers) (2).

Chemicals present in tobacco smoke that are known to be carcinogenic to animals belong to the following classes: polycyclic aromatic hydrocarbons (PAHs), aza-arenes, *N*-nitrosamines, aromatic amines, heterocyclic amines, aldehydes, inorganic compounds, and miscellaneous organic chemicals (e.g., styrene, benzene, and vinyl chloride) (2). Those for which there is sufficient evidence of carcinogenicity in humans and which are listed as Group 1 carcinogens by the International Agency for Research on Cancer include 2-naphthylamine, 4-aminobiphenyl, benzene, arsenic, chromium, and vinyl chloride. Other chemicals present that are strongly suspected of being human carcinogens are nickel, cadmium, acrylonitrile, benzo[*a*]pyrene, and polonium-210.

The constituents of tobacco smoke most likely to be responsible for tumor initiation in different organs have been deduced by consideration of the organotropism observed in occupationally induced cancers and also from the sites of tumor formation in animal studies. Thus Hoffmann and Hecht (4) propose that the likely agents responsible for tobacco-related lung and larynx cancer are PAHs, acetaldehyde, and formaldehyde (with polonium-210 as a possible minor factor); bladder cancer is attributable to aromatic amines; cancers of the esophagus and pancreas are attributable to tobacco-specific nitrosamines, while PAHs and nitrosamines are implicated in cancer of the oral cavity.

The Detection and Biological Significance of DNA Adducts

Many chemical carcinogens (and most human carcinogens) exert their biological effects through the covalent binding of electrophilic species to cellular DNA to form modified nucleotides known as adducts. Thus examination of human (or

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Abbreviations used: PAHs, polycyclic aromatic hydrocarbons; NNK, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone.

animal) DNA for the presence of DNA adducts is a means of detecting prior exposure to carcinogenic agents. Because DNA is the critical target for carcinogenesis, the formation of a DNA adduct is an event of potential biological significance. In theory, the presence of adducts in an individual indicates that the person is at risk of developing cancer, although the influence of modulating factors on the carcinogenic process will make it unlikely that the magnitude of the risk can be calculated from a single parameter such as the level of DNA adducts. Of more immediate interest is the possibility of identifying the agents present in tobacco smoke that initiate cancer in humans by characterizing the DNA adducts formed in the tissues of smokers. In recent years several sensitive methods have been developed and widely used to investigate this issue (5). Where identification of adducts has been achieved, it has often been through the use of combinations of several methods. As these methods have been described in considerable detail elsewhere, the present discussion will be confined to a few brief comments.

Immunochemical methods, employing antibodies raised against synthetic carcinogen-DNA adducts, have provided a sensitive means of detecting adducts in human tissues. Because many antibodies show a degree of cross-reactivity with adducts formed by different compounds of the same chemical class (6), this allows the general identification of adducts formed by a class, but not by a specific chemical. On the other hand, the cross-reactivity can be exploited by using immunoaffinity column chromatography to concentrate adducts from human DNA for further characterization.

An extremely versatile and sensitive method of adduct detection is the ^{32}P -postlabeling assay (7). It can be applied to the detection of many different chemically stable adducts, including those formed by large aromatic carcinogens and small aliphatic ones. The amount of information concerning the structure of the adduct is limited to its general chromatographic characteristic, from which can be inferred its apparent size, hydrophobicity, and aromaticity. Also, as described below, to some extent, the effect of variations of the assay on adduct recovery can give some information on the types of adduct being detected.

Although more selective and less sensitive than these methods, fluorescence spectroscopy provides more information on the

identity of adducts in human DNA. Clearly it is applicable only to those adducts that contain fluorescent carcinogen moieties, and this has limited its use in practice to identifying PAH-DNA adducts (8). Some methylated nucleosides are also fluorescent, but the sensitivity is often insufficient for human biomonitoring studies.

The method with the greatest potential for structural identification of DNA adducts is mass spectrometry. There are a number of cases where human carcinogen-DNA adducts have been characterized by mass spectrometry (5), although its wider application in human biomonitoring has, to some extent, been hindered by the availability of insufficient amounts of DNA in which to identify the generally low levels of adducts present. Nevertheless, improvements in sensitivity and sample derivatization are anticipated in the near future such that it will be possible to identify many more of the carcinogen-DNA adducts that are undoubtedly present in human tissues and are already detected by immunochemical and postlabeling methods. Other physical methods applied to carcinogen-DNA adduct detection include electrochemical detection and atomic absorption spectrometry (8).

Many studies of DNA adducts in human tissues have been carried out using these methods either singly or in combination. A number of questions are pertinent to studies of DNA adduct determinations in human tissues. First, what are the adducts that are being detected in the tissues? Second, with what efficiency are they detected? Third, to what extent do they present a true picture of the total DNA damage?

In most cases, these questions have only been partially answered; however, as discussed in the examples below, even the partial information obtained has been useful. As already stated, antibodies raised against a particular DNA adduct cross-react with other adducts of the same class, giving an indication of the type of adducts present in a tissue when an antigenic response is elicited. Also, the digestion, enhancement, labeling, and chromatography procedures used in ^{32}P -postlabeling determine the type of DNA adduct detected. For example, very different chromatographic procedures are used to resolve adducts formed by bulky aromatic carcinogens and those formed by simple alkylating agents. Although ^{32}P -postlabeling does not provide a direct means of identifying the DNA binding species detected, adducts can also

be characterized to a limited extent by comparison of their chromatographic mobilities with those of characterized standards (when available) and by determining the effects of different enhancement procedures on the quantity and quality of the adducts recovered. For example, the nuclease P_1 digestion method of sensitivity enhancement is suitable for PAH-DNA adducts but not aromatic amine adducts, whereas the butanol extraction method is suitable for both classes. However, neither method would be expected to detect the more polar adducts formed by tobacco-specific nitrosamines. Without synthetic standards for all the adducts detected, the efficiency of their recovery by postlabeling or their reactivity toward the antibodies used cannot be determined so that adduct levels may be underestimated; this possibility is widely recognized. To some extent, these shortcomings will be addressed as more complete characterization of adducts is achieved.

Because of the unequivocal epidemiological evidence that tobacco smoking causes cancer in a number of human tissues, monitoring DNA for the presence of smoking-related adducts by comparing samples from smokers and nonsmokers provides a means of validating the detection methods that have been described. In addition, identification of the adducts formed in different tissues will provide clues to distinguish the biologically important components of tobacco smoke that are responsible for tumor initiation in different tissues.

Smoking-related DNA Adducts in Human Tissues

There have been several ^{32}P -postlabeling studies of DNA adducts in lung tissue. A consistent finding is that levels of aromatic and hydrophobic adducts are significantly higher in smokers than in nonsmokers (9-13). In some studies, a linear correlation between estimated total or daily tobacco smoke exposure and adduct levels has been observed. However, in other studies, this correlation has not been seen. One problem with these analyses is the inevitable uncertainty regarding the accuracy of self-reporting of smoking habits by the subjects in the study population. Another study has found that adduct levels, when adjusted for total tobacco smoke exposure, are higher in women's lungs than in men's (14). Interestingly, there is some epidemiological evidence to indicate a greater risk of lung cancer for female smokers than for male smokers (15,16).

The pattern of smoking-related adducts revealed by ^{32}P -postlabeling consists of a diagonal zone of radioactivity on the 2-dimensional thin-layer chromatograms generated by resolution of the labeled digests. This is widely thought to indicate that a complex mixture of adducts results from exposure of the respiratory tract to tobacco smoke. The fact that similar levels of adducts are detected in lung tissue by both the butanol extraction and nuclease P_1 digestion procedures is an indication that PAHs are the major class of carcinogens responsible for the damage detected (11). This is supported by the detection of adducts in human lung DNA by immunoassay using antibodies that recognize PAH-DNA adducts (17). Furthermore, fluorescence spectral analysis of human lung DNA has demonstrated the presence of adducts formed by benzo[a]pyrene (18–20).

Other studies have demonstrated the presence of other, apparently smoking-related, adducts in human lung tissue. Foiles et al. (21,22) have identified by mass spectrometry the formation of 4-hydroxyl-1-(3-pyridyl)-1-butanone in hydrolysates of human smokers' lung DNA; this product results from the formation of DNA adducts by the tobacco-specific nitrosamine NNK [4-(*N*-methyl-*N*-nitrosamino)1-(3-pyridyl)-1-butanone]. Also, the analysis of bronchial DNA for the presence of 7-methylguanine has demonstrated, using ^{32}P -postlabeling, significantly higher levels in smokers than in nonsmokers (23).

Cancer of the larynx is strongly associated with tobacco smoking, and two studies have demonstrated the presence of DNA adducts in this tissue. Degawa et al. (24) showed that aromatic adducts were present in the laryngeal DNA from smokers, but not nonsmokers, and that these adducts could be detected by both the nuclease P_1 digestion and butanol extraction modifications of ^{32}P -postlabeling. Szyfter et al. (25), using the nuclease P_1 digestion procedure, demonstrated the presence of aromatic adducts in both tumor and nontumor larynx tissue, but the study population contained insufficient numbers of nonsmokers for an adequate analysis of differences in adduct levels between smokers and nonsmokers.

Studies on DNA adducts in oral mucosal cells have been conducted on various groups of tobacco users with various ^{32}P -postlabeling procedures. Dunn and Stich (26) used a procedure that involved limiting concentrations of ATP and detected adducts in cells from betel nut

chewers, Khaini tobacco chewers, and inverted smokers and also in cells from nonsmoking controls. One of the adducts was at higher levels in some of the exposed groups, while two adducts were at reduced levels. Another study of tobacco smokers, tobacco chewers, and tobacco non-users found low levels of DNA adducts in oral mucosal cells from all three groups using the butanol extraction procedure of the ^{32}P -postlabeling assay (27). None of the adducts detected were found to be specifically associated with tobacco smoking or chewing. Foiles et al. (28), using the same methods, reported that adduct levels were significantly higher in the exfoliated oral mucosal cells of smokers than of nonsmokers, although they also found that the adduct patterns of smokers and nonsmokers were qualitatively similar. In another study in which both butanol extraction and nuclease P_1 digestion methods were used, higher levels of adducts were detected by the former method, suggesting a significant contribution of aromatic amines and/or nitroaromatic compounds to DNA binding (29); this study also found that the adduct levels were significantly higher in smokers.

Another tissue in which higher levels of adducts are detected by the butanol extraction procedure than by the nuclease P_1 digestion method is the bladder (30–32). Both biopsy samples and exfoliated cells recovered from urine have been found to be suitable sources of DNA for such studies. Again, adduct levels have been found to be higher in smokers than in nonsmokers, consistent with the increased risk of bladder cancer faced by smokers. The evidence in favor of aromatic amines as the major adduct-forming class of compounds in this tissue is compatible with the finding that occupational exposure to aromatic amines has resulted in bladder cancer. Furthermore, one of the adducts detected in human bladder DNA has been found to be chromatographically indistinguishable from the major DNA adduct formed by 4-aminobiphenyl (31).

Smoking is a risk factor for cervical cancer, although epidemiologists are still divided on whether this association is real or is due to a confounding factor (33). Significantly higher levels of DNA adducts, detected by ^{32}P -postlabeling, have been found in the cervical epithelium of smokers than of nonsmokers (34,35), an observation that gives some credence to the epidemiological observations, although it does not prove a causal relationship. In these

studies significant differences in adduct levels were observed when butanol extraction was used as the enrichment procedure, but not when nuclease P_1 digestion was used (34,35). In another study where nuclease P_1 digestion was used to enhance sensitivity, elevated adduct levels were detected only in those smokers who were also users of oral contraceptives (36). As oral contraceptive use is another apparent risk factor for cervical cancer, this is an interesting observation that warrants further investigation.

Several environmental carcinogens are mammary carcinogens in experimental animals (37) and yet there is no clear evidence for the involvement of any such agent in the etiology of human breast cancer. Nevertheless, the pattern of mutations seen in the p53 gene in human breast tumors suggests the involvement of exogenous agents in inducing these mutations in a significant proportion of cases (38). Furthermore, there is a suggested association between heavy smoking from an early age and increased risk of breast cancer (39) and also between passive smoking and breast cancer (40). Both observations require confirmation. A pilot postlabeling study of DNA from breast tumor and adjacent normal tissue DNA found that an adduct pattern showing the diagonal radioactive zone characteristic of smoking-related adducts in other tissues was present in 5 of 15 samples from smokers but not in any of 8 samples from nonsmokers (41). This was statistically significant ($p < 0.01$) although there was no difference in the overall levels of adducts in smokers and nonsmokers.

There are some epidemiological studies [cited in (42)] that point to the increased risk of gastric cancer among male smokers. In support of this evidence, DNA adduct levels were found by ^{32}P -postlabeling to be significantly higher in gastric tumor tissue from male smokers than in tissue from nonsmokers (42).

For reasons that are not entirely clear, cigarette smoking is a risk factor for anal cancer (OR = 11.0; 95% CI 2.8–43.1 for men who have smoked for 30 or more years) (43). A pilot postlabeling study of DNA from anal tissue of 10 smokers and 10 nonsmokers has demonstrated that 9 of 10 of the smokers' samples produced the diagonal zone of radioactivity while none of the nonsmokers' samples did (P Skinner, A Hewer, and DH Phillips, unpublished results).

Postlabeling analysis has been carried out on human sperm DNA to determine

whether smoking-related adducts are formed in germ cells (44). No clear evidence of differences in adduct patterns among heavy smokers, light smokers, and nonsmokers was observed. It remains the case that there is no convincing evidence that smoking induces germ-line mutations in humans (45).

The studies described thus far have all involved examination of DNA isolated from target organs or from cells that are perceived to be potential target cells. In many cases, these samples are not readily obtainable from healthy individuals and require surgical or somewhat invasive procedures that preclude, for practical or ethical reasons, widespread population sampling or repeated sampling of the same individuals. Sources of human DNA that have been analyzed for adducts are listed in Table 1. For studies of occupational or environmental exposure to carcinogens (where the study populations are healthy), DNA from white blood cells is a readily

obtainable source of DNA for analysis. Many studies have demonstrated increased levels of adducts in leukocytes or lymphocytes as a result of occupational exposure to carcinogens, in particular to PAHs (32). Where investigators have compared smokers and nonsmokers, however, results have been variable. In studies of whole white blood cells and some studies of the monocyte and lymphocyte fraction (consisting mostly of lymphocytes), adduct levels in smokers and nonsmokers were the same (11,46,47). In other studies on lymphocytes and monocytes, elevated levels in smokers were detected (48,49). In one study in which DNA from the lungs and white blood cells of lung cancer patients were analyzed, no correlation between adduct levels in the two tissues was observed (50). At the same time, studies of occupational exposures to carcinogens that have taken account of dietary influences have demonstrated that putative ingestion of carcinogens in food (e.g., in barbecued meat) can result in detectable DNA adducts in blood cells (51). Thus it is clear from these studies that, while monitoring white blood cells (or the mononuclear cell fraction) for DNA adducts can in some instances provide evidence of exposure to tobacco, the confounding influence of genotoxic agents from other sources may also be observed.

Holz et al. (52) have reported that monocytes, but not unstimulated human lymphocytes, formed DNA adducts when treated separately with benzo[a]pyrene *in vitro*, but that lymphocytes incubated in the presence of monocytes did form adducts. They concluded that lymphocytes in whole blood form adducts from the carcinogen after it has been activated by other cells.

Studies of 7-methylguanine levels have demonstrated that they differ significantly between lymphocytes and granulocytes (53). Also, the mean adduct levels were higher in smokers than in nonsmokers ($p < 0.05$).

Finally, it is widely recognized that tobacco smoking during pregnancy affects the unborn child. Transplacental exposure to tobacco carcinogens is demonstrated by the detection in placental DNA of adducts derived from PAHs present in tobacco smoke (54,55).

Summary and Conclusions

The majority of investigations on the presence of DNA adducts in the target tissues of smokers either demonstrate the presence of smoking-related adducts or indicate that adduct levels are higher in smokers than in nonsmokers, although some of the adducts may be present in both groups. The presence of adducts in nonsmokers may be the result of passive smoking or of exposure to carcinogens from other environmental sources. These results reinforce the view that the carcinogenic effects of tobacco smoke are a result of the genotoxic action of some of the many carcinogens present in this extremely complex mixture.

Although most of the adducts that have been detected by methods such as post-labeling and immunoassay have not been fully characterized, some general conclusions can be made about their probable nature. Thus, most of bulky adducts detected in the respiratory tract appear to be formed by PAHs while the predominant damage in the urinary bladder probably derives from aromatic amines. Other studies have indicated adduct formation in the lung by nitrosamines, but the extent of damage relative to that caused by PAHs has not yet been determined. The presence of adducts formed by benzo[a]pyrene and NNK in lung tissue and by 4-aminobiphenyl in bladder cells has been demonstrated with reasonable certainty; it is anticipated that recent advances in the methodology for adduct detection and characterization will result in rapid progress in identifying many more of the adducts present in human DNA in the near future.

Table 1. Sources of DNA for human biomonitoring of tobacco-related cancer.

Source	Target tissue	Accessibility ^a
Peripheral blood lymphocytes	No	High
Buccal mucosa	Yes	High
Cervical epithelial cells	Yes	High
Exfoliated bladder cells	Yes	High
Hair roots	No	High
Sperm cells	No	High
Skin biopsies	No	Medium
Bladder biopsies	Yes	Medium
Lung washings or biopsies	Yes	Medium
Bone marrow cells	No	Medium
Placenta	No	Medium
Surgical specimens (various tissues)	Yes	Low
Postmortem tissue (various tissues)	Yes	Low

^aPractical or ethical ease of obtaining material from humans; consideration is also given to the possibility of obtaining samples from the same individuals on subsequent occasions.

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